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ORIGINAL ARTICLE

Genetic mapping of a barley leaf rust resistance gene *Rph26* introgressed from *Hordeum bulbosum*

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Abstract

Key message **The quantitative barley leaf rust resistance gene, *Rph26*, was fine mapped within a *H. bulbosum* introgression on barley chromosome 1HL. This provides the tools for pyramiding with other resistance genes.**

Abstract A novel quantitative resistance gene, *Rph26*, effective against barley leaf rust (*Puccinia hordei*) was introgressed from *Hordeum bulbosum* into the barley (*Hordeum vulgare*) cultivar 'Emir'. The effect of *Rph26* was to reduce the observed symptoms of leaf rust infection (uredinium number and infection type). In addition, this resistance also increased the fungal latency period and reduced the fungal biomass within infected leaves. The resulting introgression line 200A12, containing *Rph26,* was backcrossed to its barley parental cultivar 'Emir' to create an F_2 population focused on detecting interspecific recombination within the introgressed segment. A total of 1368 individuals from this F_2 population were genotyped with flanking markers at either end of the 1HL introgression, resulting in the identification of 19 genotypes, which had undergone interspecific recombination within the original introgression. F_3 seeds that were homozygous for the introgressions of reduced size were selected from each F_2 recombinant and were used for subsequent genotyping and phenotyping. *Rph26* was genetically mapped to the proximal end of the introgressed segment located at the distal end of chromosome 1HL. Molecular markers closely linked to *Rph26* were identified and will enable this disease resistance gene to be combined with other sources of quantitative resistance to maximize the effectiveness and durability of leaf rust resistance in barley breeding. Heterozygous genotypes containing a single copy of *Rph26* had an intermediate phenotype when compared with the homozygous resistant and susceptible genotypes, indicating an incompletely dominant inheritance.

Introduction

Cultivated barley (*Hordeum vulgare* L.) was one of the first domesticated cereals and served as a founder crop of modern agriculture (Park et al. 2015). Today, barley ranks fourth in global production among all cereals after maize (*Zea mays* L.), wheat (*Triticum* spp. L.) and rice (*Oryza sativa* L.) and twelfth for all crops (Food and Agriculture Organization of the United Nations 2014). In New Zealand, barley is one of the most important cereal crops. In 2016, the cultivation area of barley was the largest among all cereal crops and the production was the second highest with 364,200 metric tons (mt), following wheat (459,300 mt) (Statistics New Zealand 2016).

However, like most cultivated crops, there are various abiotic and biotic stresses, such as nutrient, water, pest and disease stress, that limit the commercial yield of barley. Barley leaf rust, caused by *Puccinia hordei*, is one of the most important diseases of barley and is widely distributed in

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major growing areas all over the world (Clifford 1985; Park et al. 2015). In recent decades, the incidence of leaf rust has increased in barley-growing regions mainly because of more intensive cultivation (Park et al. 2015).

To control the damage caused by *P. hordei*, the application of plant resistance through breeding programmes is considered the most effective method (Park et al. 2015). Host resistance to leaf rust in cultivated barley has been characterized into two types: (1) Hypersensitive or major resistance, and (2) quantitative or partial resistance (Parlevliet 2002). Hypersensitive resistance can inhibit rust development through localized cell death, and results in highly resistant infection types (IT) to barley leaf rust. In this resistance process, R genes in host plants are involved in recognition events for a corresponding avirulence (Avr) effector protein from the rust pathotype (Bettgenhaeuser et al. 2014). Thus, the hypersensitive resistance conditioned by R genes is only effective to the rust isolates carrying the compatible Avr effector proteins, making this type of resistance race-specific. In addition, the monogenic nature of hypersensitive resistance is often non-durable through the appearance of new rust pathotypes lacking the target avirulence factor (Paulitz and Steffenson 2011). Another approach to develop cultivars with more durable resistance to barley leaf rust is the use of quantitative, partial, adult plant or 'slow rusting' resistance. Unlike the complete elimination of disease symptoms by hypersensitive resistance genes, partial resistance is characterized by a susceptible infection type but coupled with a reduced rate of epidemic build-up or reduced disease severity by the pathogen (Parlevliet 2002). Partial resistance is normally conditioned by a number of quantitative trait loci (QTL) with minor effect (sometimes prefixed by *Rphq*), of which 20 QTLs have been identified from barley and its wild relatives (Qi et al. 1998, 2000; Marcel et al. 2007, 2008). The polygenic nature makes partial resistance more durable than hypersensitive resistance, but also more difficult to utilize in breeding programmes (Johnston et al. 2013).

The combination of reduced genetic diversity from intensive breeding for improved yield and quality, and the evolution of new leaf rust pathotypes, has resulted in a limit to the resistance resources available in elite barley cultivars. *Hordeum bulbosum* L., the only member of the secondary gene pool of cultivated barley, has long been investigated as a source of novel disease resistances which are not available in *H. vulgare* (Fetch et al. 2009; Jie and Snape 1989; Pickering et al. 1995, 2006; Ruge et al. 2003; Scholz et al. 2009; Shtaya et al. 2007; Toubia-Rahme et al. 2003; Walther et al. 2000; Xu and Kasha 1992). Because of incompatibility barriers between *H. bulbosum* ($2n = 2x$ or $4x$) and *H. vulgare* ($2n = 2x$), *H. bulbosum* has historically been used to produce barley double haploids through chromosome elimination (Kasha and Kao 1970). Introgression lines (ILs) with genomic segments from *H. bulbosum* introduced into *H. vulgare* genetic backgrounds were first produced from interspecific hybrids 30 years ago (Szigat and Pohler 1982) and were confirmed by using in situ hybridization and Southern blotting (Pickering et al. 1995; Xu and Kasha 1992). These ILs provide important genetic resources to explore novel traits from *H. bulbosum* and enable the utilization of diverse genes outside the primary gene pool (cultivated barley and the wild barley *H. vulgare* subsp. *spontaneum*) to improve cultivated barley (Johnston et al. 2013).

In this study, the IL called 200A12 (also coded as E-1HL in Pickering et al. 2004) was investigated, which features the very end of chromosome 1HL transferred from *H. bulbosum* into the cultivated barley genome. This introgression from the *H. bulbosum* genome was first identified in field trials as possessing a partial or 'slow rusting' resistance to *P. hordei* that is designated here as *Rph26*. In a previous study, the presence of this resistance gene in 200A12 resulted in a 16% longer latency period of *P. hordei* relative to its barley genetic background cultivar 'Emir', and 43% and 15% reductions in infection frequency compared with the cultivars 'Emir' and 'Vada', respectively (Pickering et al. 2004). A prolonged latency period was considered to be the most crucial parameter used to identify partial resistance (Neervoort and Parlevliet 1978). In addition, 200A12 had a higher percentage of early abortion and smaller established *P. hordei* colonies than 'Vada' and 'Emir', indicating a higher degree of partial resistance. Early aborted colonies were normally associated with the defence mechanism by blocking rust haustorium formation at penetration sites on plant cell walls, but not hypersensitivity (Pickering et al. 2004).

The overall goal of this project was to develop an F_2 population from a backcross between 200A12 and its own barley parental cultivar 'Emir'. This population was used to first identify interspecific recombinant genotypes within the original introgression for genetic mapping of *Rph26* and second to determine the genetic inheritance of the resistance gene.

Materials and methods

Plant and pathogen material

The line 200A12 was developed (Fig. 1) from a cross between the tetraploid *H. bulbosum* genotype A17 (from the Botanical Garden of Montevideo, Uruguay, Scholz et al. 2009) (BBBB) and the diploid barley cultivar 'Emir' (VV) to produce a partially fertile triploid hybrid (VBB) designated 178M, where V and B represent the genome constitution of *H. vulgare* and *H. bulbosum*, respectively. This triploid hybrid was then used as the pollen parent to backcross with the same barley parent 'Emir'. From the resulting progeny, the diploid 200A12 was identified as possessing resistance

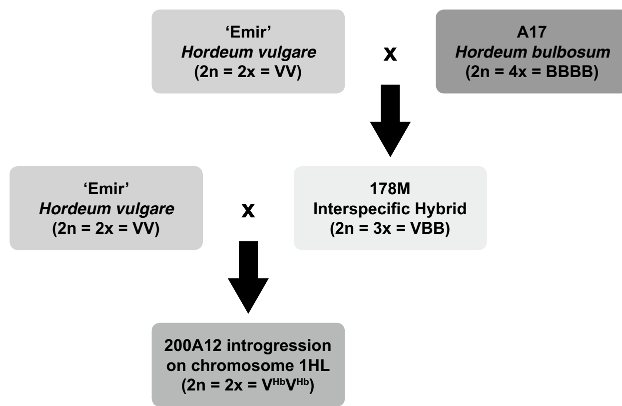

Fig. 1 Crossing plan showing the development of the introgression line (IL) 200A12 (where V and B refer to haploid genome equivalents (seven chromosomes) of *Hordeum vulgare* and *Hordeum bulbosum*, respectively)

to leaf rust and a homozygous introgression from *H. bulbosum* on the distal end of chromosome 1HL (Pickering et al. 2004). The cultivar 'Emir' is also thought to carry the adult plant resistance (APR) gene *Rph20* (5HS) from molecular marker analysis (bPb-0837-PCR) performed by Hickey et al. (2012).

A New Zealand *P. hordei* isolate, pathotype (pt) 5457P+, was used for all phenotypic evaluations of 'Emir', 200A12 and recombinant genotypes against barley leaf rust. This isolate was evaluated in a separate pathotyping test against different genotypes (Park 2003) to be virulent upon the resistance genes *Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*, *Rph19* and *RphP*.

Development of interspecific markers

Based on the *H. bulbosum*-specific single nucleotide polymorphisms (SNPs) identified from 200A12 (Wendler et al. 2015), 12 cleaved amplified polymorphic sequences (CAPS), 28 high-resolution melting (HRM) and four size-polymorphic markers within the introgressed region were developed to discriminate between *H. vulgare* and *H. bulbosum* alleles (Kong 2015). By including the previously developed markers KS240con, H31_5700 (Johnston, unpublished) and H31_14212 (Johnston et al. 2009), a total of 47 interspecific markers located at the distal end of chromosome 1HL were used to map the resistance gene *Rph26*, in 200A12.

Marker names, marker types, primer sequences and variations from the basic polymerase chain reaction (PCR) conditions can be found in Supplementary Table 1. Size-polymorphic and CAPS markers were amplified in a 10-μL reaction volume which contained 1 X ReddyMix PCR buffer (Thermo Fisher), 0.2 mM dNTPs (Fermentas), 2.5 mM $MgCl_2$ (Thermo Fisher), 0.3 μM each primer (Bioneer), 0.2 U ThermoPrime *Taq* DNA polymerase (Thermo Fisher) and 20 ng DNA template. The basic PCR conditions were 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min. HRM markers were also amplified using a 10-μL reaction volume with 2 μL 5X HOT FIREPol® EvaGreen® HRM mix-no ROX (Solis BioDyne), 0.25 μM each primer and approximately 20 ng DNA template. To prevent evaporation losses, 20 μL of PCR-grade mineral oil (SIGMA) was added to each reaction. Basic touchdown PCR conditions were 95 °C for 15 min, 10 cycles of 95 °C for 30 s, 65 °C for 30 s reducing 1 °C per cycle and 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final cycle of 95 °C for 30 s and 28 °C for 30 s to maximize the formation of heteroduplex PCR products if the DNA sample was heterozygous. All PCRs were conducted on either a C1000 Thermal Cycler (BioRad) or a Mastercycler Pro S (Eppendorf).

Population development for gene mapping and determining genetic inheritance

To map the resistance gene, *Rph26*, within the introgressed segment and determine its genetic inheritance, the IL 200A12 was backcrossed to its own barley parent 'Emir' to produce F_1 seeds. The F_1 plants were screened with the marker H31_14212 to confirm that they were heterozygous for the 1HL introgression from 200A12. F_1 plants with the correct genotype were allowed to self-fertilize to produce seeds for the F_2 mapping population (377B population).

As the barley genetic background cultivar 'Emir' was consistent throughout the development of this population, all subsequent mapping was focused solely on interspecific recombination events within the original *H. bulbosum* introgression on chromosome 1HL. Genomic DNA was extracted from a total of 1368 F_2 plants from freeze-dried leaf material using the 'Wheat and Barley DNA Extraction in 96-well plates' method (Chao and Somers 2012). The F_2 population was then genotyped with the markers CM_1186 and H31_14212, which were located at the proximal and distal ends of the introgression, respectively. Interspecific recombinant genotypes were identified when F_2 individuals had different genotypes for the markers CM_1186 and H31_14212. F_3 seeds from each F_2 recombinant genotype were obtained via self-fertilization. To allow identification of homozygous (true breeding) F_3 recombinant genotypes prior to seed germination, endosperm powder was scraped from between 20 and 40 F_3 seeds per F_2 recombinant with a scalpel blade and genomic DNA was extracted using a quick seed extraction protocol (von Post et al. 2003). The DNA from each F_3 seed was screened with the same flanking markers to identify the seeds which were homozygous for each interspecific recombination event. The identified F_3 homozygous recombinant seeds from each F_2 recombinant

genotype were retained for pathological and molecular marker screening. In this way, pathology and genotyping were simplified by using F_3 homozygous recombinant lines instead of F_2 heterozygous recombinant genotypes and the time period used to obtain homozygous recombinant lines for genetic mapping was shortened.

To determine the genetic inheritance of *Rph26*, a separate set of 60 F_2 plants derived from the 377B population ('Emir' × 200A12) was marker genotyped with CM_1186 and H31_14212, using the same DNA extraction method by Chao and Somers (2012). These markers identified which plants had two copies (BB), one copy (VB) or none (VV) of the *H. bulbosum* introgression on chromosome 1HL.

Inoculation and evaluation of infection

Two separate experiments were conducted under greenhouse conditions to evaluate the phenotypic response of these genotypes to inoculation with *P. hordei*. The first experiment was used to determine the genetic location of *Rph26* within the *H. bulbosum* introgression on chromosome 1HL. Homozygous F_3 seeds from recombinant lines identified in the marker testing of seed DNA extracts plus parental lines were pre-germinated on damp filter paper in petri dishes at 4 °C for 1 week and then transferred to room temperature. Once germinated, three healthy-looking F_3 seedlings per F_2 recombinant were transferred into 15-cm-diameter pots containing potting mix, with one seedling per pot. The layout of the experiment was a 2-latinized row–column design generated using CycDesign 5.1 (VSN International Ltd 2013) and consisted of three replicates of 28 plants (4 × 7) featuring one plant (pot) each of 'Emir', 200A12 and 18 of the homozygous F_3 recombinant lines (but only two replicates of 377B_708_F_3). Owing to an unusual genotyping result in F_2 and F_3 marker testing for 377B_794, eight plants per replicate were included. When most plants in the experiment reached tillering stage (growth stage (GS) 21, Zadoks et al. 1974), leaf rust inoculations were carried out for three successive days, with a complete replicate inoculated each day. Prior to inoculation, the third leaf of each plant, counted from the bottom, was folded down over a plastic board and secured using adhesive tape. Water agar slides (2%) were evenly distributed on the plastic board, with one slide placed between every four secured leaves, to estimate urediniospore densities and germination rate. Leaf rust inoculations were performed by atomising a urediniospore–mineral oil suspension (1 mg of urediniospores in 2 mL of mineral oil (Pegasol, Mobil Oil) per eight plants) over the secured plant leaves. After incubation at 20 °C and 100% relative humidity in the dark for 24 h, the plants were maintained at 22 °C, 14/10 h light/dark photoperiod in a greenhouse. The urediniospore densities (urediniospores/cm^2) and germination rate (%) were estimated by counting the number of urediniospores (germinated and non-germinated) within a 1 cm^2 area of water agar slides under a compound microscope. The urediniospores with germ tubes which were equal to or greater than the width of the urediniospores were determined as germinated.

For the phenotypic evaluation of the F_3 recombinants, leaf rust infection was assessed by visually counting the number of uredinium, by observing infection type (IT) and by using a quantitative method of assessing fungal growth in the infected leaf tissues (Ayliffe et al. 2014). At 4–5 days post-inoculation (DPI) before mature uredinia appeared, a 5-cm long area of each inoculated leaf containing pale flecks, which indicated the early signs of uredinia formation or resistance response, was selected and marked. Once visible, the mature uredinia within the marked areas were counted daily until 14 DPI, at which point no further uredinia developed. The IT was assessed at 10 DPI using a 0–4 scale as described by Park (2003). Inoculated plants with ITs of three and above were considered to be compatible (i.e. virulent pathogen/susceptible host). Once the uredinium count was completed, the marked 5-cm-long leaf segments were collected, and all the replicated leaf tissues from a same test line were pooled and used for evaluation of fungal biomass using the protocol described by Ayliffe et al. (2014). Briefly, samples were extracted in 1M KOH containing 0.1% (vol/vol) Silwet L-77 by autoclaving at 121 °C and 15 psi for 20 min. The leaf tissue was washed and neutralized with 50 mM Tris-HCl (pH 7.0), and then immersed in Tris buffer at a rate of 8 ml of 50 mM Tris-HCl (pH 7.0) per 1 g fresh leaf tissue for sonication. Three replicate aliquots of 200 µL per sample were each mixed with 10 µL of a 1 mg/mL solution of lectin wheat germ agglutinin conjugated to fluorescein isothiocyanate (WGA-FITC, Sigma Aldrich). After staining for 10 min, the samples were centrifuged at 600×*g* for 3 min. The pelleted tissue was washed in 50 mM Tris-HCl (pH 7.0) and centrifuged twice more before being resuspended in 100 µL of 50 mM Tris-HCl (pH 7.0). Fluorescence units (FU) were measured using a SpectraMax M2 (Molecular Devices) under 485 nm absorption and 535 nm emission wavelengths at 1.0 s measurement time in black 96-well microtiter plates.

The second experiment was established to test the genetic inheritance of *Rph26* and to re-examine the phenotypes of key lines 377B_348_F_3 and 377B_708_F_3 from the first experiment using the visual assessments only. Plants were raised in 20-cm-diameter pots containing potting mix, with four plants of the same genotype per pot, resulting in three replicates of nine pots featuring one pot of the BB genotype, two or three pots of the VB genotype, one or two pots of the VV genotype, one pot each of 377B_348_F_3 and 377B_708_F_3 and two parental controls ('Emir' and 200A12). Pots were arranged randomly within each replicate. Inoculations were carried out when most plants had their flag leaf visible on

the main stem (GS37, Zadoks et al. 1974) by using the same inoculation techniques described above (but with 2 mg of urediniospores in 3 mL of mineral oil per replicate). The same method for estimating urediniospore deposition and germination, and incubation conditions was used. The observed mean germinated spore deposition rates for each experimental replicate were 98, 107 and 129 per cm^2.

Genotyping and linkage mapping

Genomic DNA was extracted from freeze-dried leaf material from one homozygous F_3 recombinant line of the three used in the pathology experiment using the DNeasy Plant Mini Kit (Qiagen). These nineteen homozygous F_3 recombinants (one from each F_2 interspecific recombinant) collectively known as "200A12_F_3_Popn", and parents, were genotyped with 47 interspecific markers to determine the extent of the *H. bulbosum* introgression in each recombinant line. A genetic linkage map was developed using the R package ASMap (Taylor and Butler 2017; version 0.4) and run through RStudio (version 1.0.136; R version 3.3.3). The genotype data for all 1368 F_2 plants were used in the genetic mapping. This was done by imputing the non-recombinant F_2 genotypes [either homozygous *H. vulgare* (VV), homozygous *H. bulbosum* (BB) or heterozygous (VB)] for all markers using only the data obtained from the flanking markers. For example, plants that were genotyped as VB for CM_1186 and VB for H31_14212 were assumed to be VB for all of the intervening markers. The reconstituted F_2 genotypes for the detected interspecific recombinants were included in the F_2 mapping by combining the genotypes obtained from the F_2 flanking marker screening with the genotyping results of the F_3 homozygous recombinant lines for all the remaining markers (Fig. 2). This mapping scheme both reduced the workload and cost associated with genotyping the entire F_2 population (of which the majority were non-recombinant) and enabled the genetic distance between markers to remain accurate for the complete mapping population. QTLs representing five pathological traits (mean uredinium count 6 DPI, mean uredinium count 14 DPI, mean time, latency period and fungal biomass) measured on the homozygous F_3 recombinant lines ('200A12_F_3_Popn') were mapped onto the F_2-derived map using the R package "qtl" (Broman et al. 2003), with population type 'dh' (doubled haploid) to represent the homozygous F_3 lines and the function 'scanone' using Haley–Knott regression with the calc.genoprob parameters of step = 0.01 cM and error.prob = 0.001. Logarithm of the odds (LOD) significance thresholds were set at alpha = 0.01 with 10,000 permutations.

Statistical analysis

Analyses of uredinium counts at selected assessments

The uredinium count data were separately analysed at two time points (6 and 14 DPI for the first experiment and 7 and 13 DPI for the second experiment) using a hierarchical generalized linear model approach (HGLM, Lee et al. 2006), with a Poisson distribution for the fixed effects (lines) and a gamma distribution for random effects (replicates), and logarithmic links for both. The dispersion was estimated for both assessment dates. The importance of the random effects was assessed with a χ^2 test of the change in deviance on dropping the term, as implemented in GenStat's HGRTEST procedure (VSN International Ltd 2015). An overall test for differences between the lines was carried out similarly, using GenStat's HGFTEST procedure. In the results, means and associated 95% confidence limits are presented. These were obtained on the link (logarithmic) scale and back-transformed for presentation.

Modelling the distribution of time until uredinium formation

The number of uredinia at each assessment included both newly formed uredinia and the existing uredinia from the previous time point. Thus, the data are in the form of 'accumulated counts' (O'Neill et al. 2004; Hunter et al. 1984) and are suitable for analysis by methods that estimate the distribution of times until uredinium formation (DPI for each

Marker name (proximal to distal)

Line	Genotype	CM_1186	KS240con	CM_1187	CM_1194	CM_1144	CM_1143	CM_1191	CM_1189	CM_1192	CM_1201	CM_1167	CM_1150	CM_1148	CM_1200	CM_1193	CM_1160	CM_1206	CM_1155	CM_1154	CM_1156	CM_1157	CM_1202	CM_1198	H5700	CM_1195	CM_1197	CM_1163	CM_1199	H31_14212
377B_1079_F_2	F_2 Flanking Markers	BB	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	??	VB
377B_1079_F_3	F_3 Genotype	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1079_F_2	Reconstituted F_2 Genotype	BB	BB	BB	BB	BB	BB	BB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB	VB

Fig. 2 An example of how the reconstituted F_2 genotypes were derived from the flanking marker data obtained during the F_2 screening and the final F_3 homozygous genotyping of all markers (BB, VV, VB and ?? denote a homozygous *H. bulbosum*, a homozygous barley genotype, a heterozygous genotype and the absence of genotyping, respectively)

uredinium to appear), as described in O'Neill et al. (2004). The analysis was carried out in two parts: Firstly, the total numbers of uredinia per line at each assessment were analysed. Secondly, the total numbers of uredinia per line in each replicate and during each assessment were analysed. When combined with the results from the first analysis, this second analysis allowed an assessment of whether there was substantial variation between replicates within lines. The data were modelled using the logistic distribution of the log DPI, as implemented in GenStat's CUMDISTRIBUTION procedure (Butler et al. in VSN International Ltd 2015). It was assumed that no further uredinia would be formed after the final assessment (14 DPI; 'ALLRESPOND = yes' selected for the fitting process). The cumulative distribution function (c.d.f) is $F(z)$:

$$F(z) = \frac{1}{1 + e^{(-b\times(z-m))}}$$

where $z = \log(\text{DPI–Lag})$ and b, m, Lag are estimated parameters.

The primary results of these analyses were estimates of the lag before uredinia began to appear (Lag), the mean time to uredinium formation (Mean), the DPI to 50% uredinium formation (median or latency period, LP) and the standard deviation of the distribution of DPI to uredinium formation (SD). Mean, LP and SD were calculated from Lag, b and m, as follows:

$$\text{Mean} = \text{Lag} + \frac{\pi}{b} \times \frac{e^m}{\sin(\pi/b)}$$

$$\text{LP} = \text{Lag} + e^m$$

$$\text{SD} = e^m \times \sqrt{\frac{\pi}{b \times \sin(\pi/b)} \times \left(\frac{1}{\cos(\pi/b)} - \frac{\pi}{b \times \sin(\pi/b)}\right)}$$

Note that if $\tan(\pi/b) < (\pi/b)$, the SD could not be calculated (since it would require the calculation of the square root of a negative number). Some 'analysis of parallelism' (Ross 1984) was carried out to assess whether any of the parameters b, m or Lag varied substantially between lines. The analyses were all carried out with GenStat (Payne et al. 2015).

Results

Genetic mapping of *Rph26*

Development of '200A12_F3_Popn'

After screening with the flanking markers CM_1186 and H31_14212, 19 F_2 interspecific recombinant genotypes were identified from a total F_2 mapping population of 1368 plants. One of these genotypes, 377B_794, was detected as displaying a homozygous *H. bulbosum* genotype for the proximal marker and a homozygous *H. vulgare* genotype for the distal marker as an F_2 plant, indicating that it was the rare product of two recombinant gametes. Marker testing of the F_3 seed DNA identified between two and twelve F_3 homozygous recombinant seeds from each of the F_2 recombinant genotypes (8–45% of tested progeny, Mean = 24.5%).

Phenotypic evaluation

Visual assessment The mean number of uredinia varied among lines at both assessments; 'Emir' had the highest number of uredinia, at 55 and 77 per plant (6 DPI and 14 DPI, respectively), whilst 200A12 had one of the lowest, at 2 and 15 (6 DPI and 14 DPI, respectively). Uredinium numbers of the 377B_794_F_3 plants tended to be at the bottom half of the range of numbers, whilst numbers for the other recombinants covered the whole range (0.4–50.8 at 6 DPI and 9.0–65.1 at 14 DPI, Table 1). The final number of uredinia (14 DPI) was very highly correlated with the numbers at 6 DPI ($r = 0.96$ for individual pot data and $r = 0.97$ for the means).

Using the ITs alone (Table 1), it was possible to classify all the '200A12_F_3_Popn' lines into two qualitative groups: one group having ITs similar to the resistant parent 200A12, with small- to medium-sized uredinia surrounded by chlorotic and/or necrotic tissue (Fig. 3b), and another group having infection types similar to the susceptible parent 'Emir', with medium to large uredinia with or without surrounding chlorosis (Fig. 3a). The exception was line 377B_708_F_3 which was considered to be an intermediate type, with IT similar to 200A12 but with higher uredinium counts at both time points (Table 1).

Statistical modelling The model which estimated different values for all three of the main parameters (Lag, b, m) fitted substantially better than models with either a single Lag, b or m parameter ($\chi^2_{30} = 54.2$, $p = 0.004$; $\chi^2_{30} = 51.7$, $p = 0.008$; $\chi^2_{30} = 117.7$, $p < 0.001$, respectively). The model-derived estimates for mean time and latency period differed strongly between the parental lines 'Emir' (shorter mean time and latency period) and 200A12 (longer mean time and latency period) (Fig. 4), with the results from the entire '200A12_F_3_Popn' forming a continuous distribution (Table 1).

Fungal biomass assay As the inoculated leaf tissue from each line was pooled across the biological replicates, only the mean FU and standard deviation for the three technical replicates are shown (Table 1). The resistant parent 200A12 and susceptible parent 'Emir' gave clearly differ-

Table 1 Phenotypic response data (first experiment) from '200A12_F_3_Popn' and parental lines to *Puccinia hordei*

Line	Mean Uredinium number (6 dpi)		Mean Uredinium number (14 dpi)		Mean Time		Latent Period		n	Infection type 10dpi			Resistance Assignment	Fungal Biomass (Pooled FU)	
		(95% confidence limits)		(95% confidence limits)		(standard error)		(standard error)		Rep1	Rep2	Rep3			(standard deviation)
377B_311_F_3	14.4	(5.3,39.2)	29.0	(14.0,59.9)	6.80	(0.38)	6.03	(0.14)	3	2+C	2-	2-C	R	537	(34)
377B_348_F_3	4.9	(0.9,26.3)	19.3	(8.1,46.3)	6.69	(0.17)	6.50	(0.16)	3	2-	2	2	R	458	(14)
377B_794_F_3[+]	5.5	(2.7,11.2)	17.6	(9.7,32.0)	6.99	(0.29)	6.45	(0.18)	24	2 2- 2-C 2+C 2+C 2- 2C 2 2-C 2-C 2- 2- 2 2-C 0C 2- 2-C 2- 2- 2- 2-C 2 12- 2-			R	569	(34)
377B_953_F_3	4.8	(0.8,27.3)	19.9	(8.2,48.6)	7.14	(0.25)	6.90	(0.21)	3	2	1	2-	R	429	(33)
377B_1073_F_3	9.0	(2.2,37.0)	23.0	(9.3,56.6)	7.52	(0.61)	6.54	(0.20)	2	2-	2C	dead	R	577	(33)
377B_1079_F_3	10.4	(3.5,31.6)	16.6	(6.9,39.8)	6.11	(0.19)	5.81	(0.12)	3	2-NC	2+	2-	R	394	(14)
377B_1120_F_3	11.1	(3.8,32.0)	25.6	(12.4,52.8)	6.68	(0.22)	6.19	(0.11)	3	2-C	2-	2+	R	537	(3)
377B_1122_F_3	2.2	(0.2,23.3)	7.7	(2.1,27.5)	7.73	(0.87)	6.66	(0.32)	3	2-	2-C	0	R	374	(17)
377B_1296_F_3	4.2	(0.7,25.7)	17.2	(6.9,43.3)	7.36	(0.35)	6.83	(0.24)	3	2-	2-	2-C	R	467	(24)
377B_1325_F_3	6.3	(1.4,28.9)	19.5	(7.9,48.1)	6.80	(0.25)	6.37	(0.16)	3	2	2-C	2-	R	520	(30)
377B_1365_F_3	0.4	(0.0,*)	10.0	(2.9,34.3)	9.10	(0.38)	8.83	(0.34)	3	2-	2-	2-	R	343	(10)
200A12	2.0	(0.2,25.1)	15.0	(5.7,39.5)	7.77	(0.44)	7.10	(0.23)	3	2-C	2	2-	R	533	(41)
377B_708_F_3	17.7	(6.2,50.5)	42.9	(21.3,86.5)	6.32	(0.12)	6.14	(0.11)	2	NA	2+	2	??	744	(43)
Emir	55.0	(32.5,93.2)	77.3	(48.8,122.4)	5.92	(0.07)	5.64	(0.05)	3	3+	3+C	3+C	S	816	(23)
377B_713_F3	20.9	(9.8,44.9)	32.8	(17.6,61.3)	6.14	(0.18)	5.69	(0.10)	3	3+	3-	3C	S	714	(8)
377B_811_F_3	24.7	(11.0,55.3)	35.4	(17.7,70.6)	6.08	(0.25)	5.56	(0.10)	3	3	3	3	S	681	(13)
377B_935_F_3	40.7	(22.0,75.4)	55.2	(31.7,96.2)	5.71	(0.06)	5.57	(0.07)	3	3	3-	3+C	S	934	(8)
377B_1262_F_3	31.7	(16.0,62.9)	51.4	(29.0,91.0)	5.87	(0.06)	5.79	(0.06)	3	3	3	3+C	S	790	(16)
377B_1270_F_3	37.2	(19.7,70.4)	54.3	(31.4,94.0)	6.02	(0.13)	5.62	(0.07)	3	3	3+	3	S	970	(25)
377B_1287_F_3	36.2	(19.5,67.3)	50.4	(29.5,86.2)	5.91	(0.10)	5.62	(0.07)	3	3	3+	3+	S	849	(52)
377B_1323_F_3	50.8	(27.6,93.7)	65.1	(37.6,112.7)	5.68	(0.05)	5.60	(0.06)	2	dead	3+C	3+C	S	967	(23)

Infection types (IT) to *P. hordei* were assessed at 10 days post-inoculation (DPI). Lines 377B_348_F_3 and 377B_708_F_3 gave identical marker genotypes and are highlighted in grey on account of discrepancies in their mean uredinium (pustule) counts and fungal biomass data. n = number of replicate plants per genotype. Infection types: 0 = no visible symptoms, 1 = minute uredinia surrounded by mainly necrotic tissue, 2 = small to medium uredinia surrounded by chlorotic and/or necrotic tissue, and 3 = medium to large uredinia with or without surrounding chlorosis. The letters 'C' and 'N' indicate greater than normal chlorosis or necrosis, respectively; '–' or '+' indicates lower or higher infection types than normal, respectively (Park 2003)

FU fluorescence units

*Note that the upper confidence limit for a very small mean is often inaccurate so is not shown

+Due to the similarity of the results from the 377B_794 plants and the complexity of deriving means from the model analysis, mean time and latency period data are shown for the median plant only

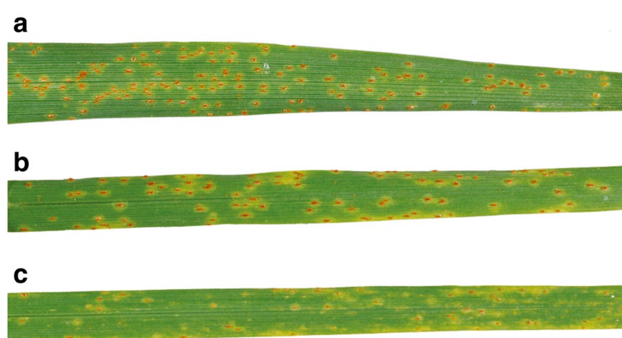

Fig. 3 Typical examples of leaf rust (*Puccinia hordei* pathotype 5457P+) infection on the second leaf of barley seedlings 10 days after inoculation. **a** The susceptible control line 'Gus', **b** 'Emir' (*rph26*) and **c** 200A12 (*Rph26*)

ent mean FU at 533 and 816, respectively, with the mean FU for all of the recombinant lines falling between 343 and 970 (Table 1). The assignment of the recombinant lines into resistant or susceptible groups based only on IT (Table 1) was also reflected in the mean fungal biomass data (resistant < 580 FU and susceptible > 680 FU), with the exception of line 377B_708_F_3, which gave a resistant IT but a high mean fungal biomass of 744.

Genetic linkage map

From the 47 interspecific markers used for genotyping, a total of 29 markers consisting of 14 CAPS, 11 HRM and four size-polymorphic markers gave clear and consistent genotype results (Table 2). By combining the flanking marker genotypes of the F_2 interspecific recombinants (CM_1186 and H31_14212) and the genotyping results of '200A12_F_3_Popn' for the remaining markers (from KS240con to CM_1199 in Table 2), the genotypes of F_2 interspecific recombinants for the remaining markers were reconstituted.

The genetic linkage map of the *H. bulbosum*-introgressed region of 200A12 was constructed by using the reconstituted

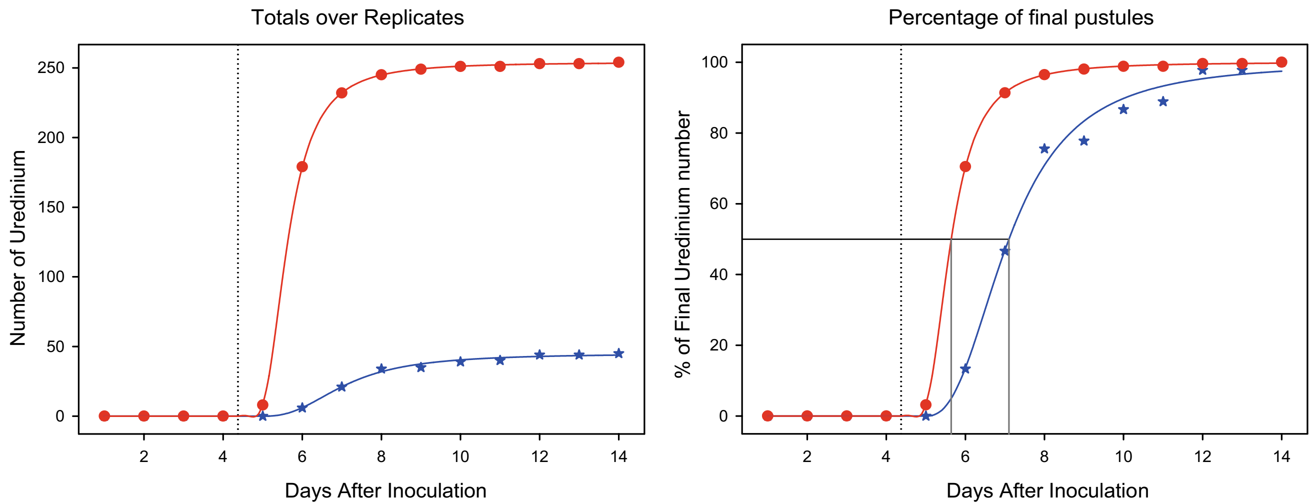

Fig. 4 Statistical modelling of uredinium (pustule) development in the parental lines 'Emir' (red circles) and 200A12 (blue stars) combined from all three replicate plants. The left-hand figure shows a distinct difference in the cumulative number of uredinium between the susceptible parent 'Emir' (red circles) and the resistant parent 200A12 (blue stars). The right-hand figure shows the percentage uredinium development, with uredinia developing more rapidly on 'Emir' (red circles, shorter latency period) than on 200A12 (blue stars, longer latency period). The vertical dotted line shows the end of the lag phase. The solid black lines indicate the appearance of 50% of eventual uredinium number, thus indicating the latency period (median time to uredinium formation) (color figure online)

Table 2 Genotyping results from the '200A12_F_3_Popn' and parental lines indicating the relative size and position of each recombinant line within the original introgression located in 200A12

		Marker name (proximal to distal)																												
Line	Resistance Assignment	CM_1186	KS240con	CM_1187	CM_1194	CM_1144	CM_1143	CM_1191	CM_1189	CM_1192	CM_1201	CM_1167	CM_1150	CM_1148	CM_1200	CM_1193	CM_1160	CM_1206	CM_1155	CM_1154	CM_1156	CM_1157	CM_1202	CM_1198	H5700	CM_1195	CM_1197	CM_1163	CM_1199	H31_14212
200A12	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_311_F_3	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1073_F_3	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1120_F_3	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1325_F_3	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1365_F_3	R	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_794_F_3	R	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_953_F_3	R	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1079_F_3	R	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1122_F_3	R	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1296_F_3	R	BB	BB	BB	BB	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_713_F_3	S	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_1262_F_3	S	BB	BB	BB	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
377B_348_F_3	R	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_708_F_3	??	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_1270_F_3	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_935_F_3	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_1323_F_3	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_811_F_3	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
377B_1287_F_3	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
Emir	S	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV
Recombination Events		0	0	4	0	0	0	5	0	1	0	2	0	0	1	3	2	1	0	0	0	0	0	0	0	0	0	0	0	

Rph26

V and B represent the haploid marker genotypes of *Hordeum vulgare* and *Hordeum bulbosum*, respectively. Recombination events between pairs of neighbouring markers are listed at the bottom of the table. The bar at the bottom of the table indicates the outer limits of the *Rph26* locus

genotypes from the nineteen F_2 interspecific recombinants, combined with the remaining 1349 non-recombinant F_2 genotypes (homozygous *H. vulgare*, homozygous *H. bulbosum* or heterozygous for all markers) using the R package ASMap (Taylor and Butler 2017). The barley leaf rust resistance, *Rph26*, was mapped as five separate QTLs (uredinium number 6dpi and 14dpi, mean time, latency period and fungal biomass) within the *H. bulbosum* introgression

on chromosome 1HL using the results of the pathological evaluation (first experiment). QTL peaks for all traits were significant at the alpha = 0.01 level (LOD thresholds were 2.7, 3.0, 2.5, 2.0 and 2.8, respectively) and the QTL peak for all five pathological traits was located at 0.10–0.11 cM, proximal of a cluster of four markers CM_1143, CM_1144, CM_1191 and CM_1194 (Fig. 5).

Analysis of the allelic effects for all five quantitative traits, at the near-peak marker CM_1194, revealed a clear separation between ‘Emir’ and 200A12 genotypes (data not shown). However, the data for line 377B_708_F_3 gave inconsistencies between the resistant IT scores and the more susceptible values for uredinium count at 14 DPI and fungal biomass.

Genetic inheritance of *Rph26* In order to determine the genetic inheritance of *Rph26*, a set of 60 F_2 plants from the 377B population were genotyped using the same flanking markers CM_1186 and H31_14212. These markers identified 12 plants homozygous for the *H. bulbosum* introgression (377B_BB), 32 heterozygous plants (377B_VB) and 16 plants without the introgression (377B_VV). Inoculations at flag leaf emergence with *P. hordei* revealed an intermediate uredinium number for the heterozygous (377B_VB) genotypes when compared with the homozygous presence (377B_BB) and absence (377B_VV) genotypes (Table 3). The additional lines 377B_348_F_3 and 377B_708_F_3 gave responses similar to the resistant parent in the first two replicates; however, 377B_708_F_3 gave a susceptible response in the third replicate (Fig. 6), resulting in a higher overall mean (Table 3). The third experimental replicate gave higher uredinium counts for both of the susceptible genotypes 377B_VV and ‘Emir’ (Fig. 6) which was reflected in the higher mean germinated spore deposition rate for the third replicate.

Discussion

Access to new sources of genetic disease resistance is key to the future of barley breeding. *H. bulbosum*, as the sole member of the secondary genepool of *Hordeum*, is an important donor of disease resistance (and potentially other traits) for cultivated barley. However, moving traits from *H. bulbosum* into barley is not trivial and is exacerbated by suppressed interspecific recombination and the

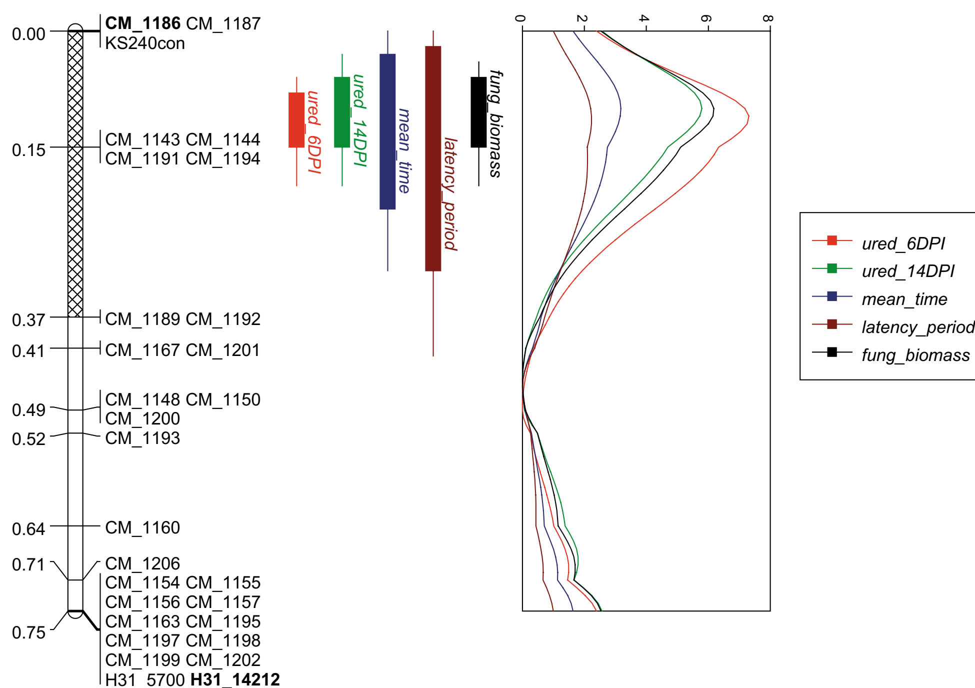

Fig. 5 Genetic linkage map of the *Hordeum bulbosum* introgression within 200A12 on barley chromosome 1HL. DNA markers CM_1186 and H31_14212 (bold) were the flanking markers used to detect interspecific recombinants. The graphs on the right-hand side show LOD scores for five pathological traits; mean uredinium number at six (‘ured_6DPI’, red) and fourteen (‘ured_14DPI’, green) days post-inoculation (DPI), mean time (‘mean_time’, dark blue), latency period (‘latency_period’, brown) and fungal biomass (‘fung_biomass’, black). The thick, vertical QTL bars indicate the area covered by a drop of one LOD from the QTL peak, and the thin vertical lines, a drop of two LOD. The crosshatched region indicates the likely position of *Rph26*. Numbers to the left of the linkage map indicate genetic distance (cM) from the most proximal marker (CM_1186). Linkage map and QTL graphs were constructed using MapChart v2.2 (Voorrips 2002) (color figure online)

Table 3 Mean number of uredinium per genotype (second experiment) across all replicates for selected days after inoculation (95% confidence limits) and estimated parameters (with standard error) for the distributions of times until uredinium appears, for each genotype, estimated from the total uredinia over replicates for each genotype at each assessment

Line	Mean uredinium number (7dpi)		Mean uredinium number (13dpi)		Mean time		Latency period	
200A12	15.8	(4.8,51.8)	45.4	(22.8,90.5)	9.3	(1.67)	8.0	(0.57)
377B_BB	3.6	(0.8,17.6)	55.6	(31.3,99.0)	9.3	(0.64)	8.7	(0.41)
377B_VB	37.0	(18.1,75.7)	114.0	(83.9,154.8)	8.9	(0.40)	8.0	(0.18)
377B_VV	39.6	(20.5,76.3)	176.1	(126.0,246.1)	8.1	(0.31)	7.5	(0 15)
Emir	133.9	(66.9,268.1)	244.4	(168.5,354.6)	7.6	(0.25)	7.2	(0.13)
377B_348_F_3	1.4	(0.1,23.0)	36.9	(16.3,83.4)	9.2	(0.69)	8.8	(0.52)
377B_708_F_3	50.7	(22.0,116.9)	107.6	(64.4,179.7)	8.3	(0.77)	7.4	(0.27)
	(95% confidence limits)		(95% confidence limits)		(Standard error)		(Standard error)	

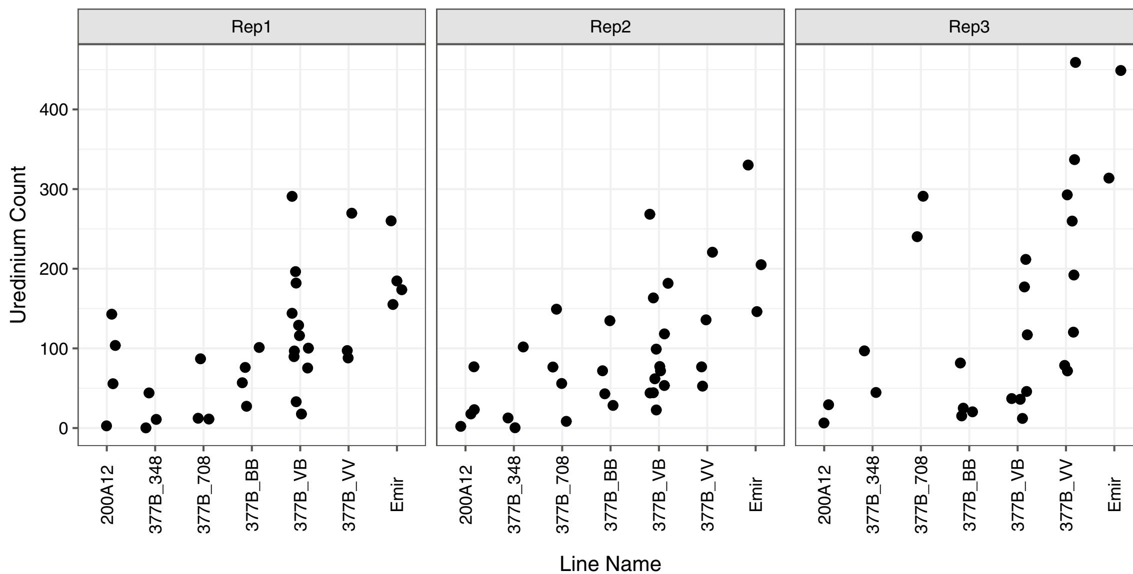

Fig. 6 Uredinium counts made 13 days post-innoculation for the second phenotypic experiment across the three experimental replicates (Rep1, Rep2 and Rep3). A small amount of horizontal spread (jitter) has been applied to the data points to allow all points to be seen

linkage drag of undomesticated alleles. In this study, the quantitative leaf rust resistance gene, *Rph26*, was genetically mapped within the *H. bulbosum* introgression on 1HL in the line 200A12 (also coded as E-1HL, Pickering et al. 2004). This not only helps to identify markers closely linked to *Rph26*, but also reduces the size of the introgression around the trait of interest and hence reduces the potential for linkage drag. A major impediment to working with a wild species such as *H. bulbosum* is usually the lack of marker resources. This study benefitted considerably from the availability of a large number of SNPs identified in genotype by sequencing (GBS) experiments between a set of introgression lines and their barley parents (Wendler et al. 2015). This sequence resource enabled markers to be quickly developed, with good coverage over the entire introgressed region on 1HL in 200A12 (Kong 2015).

There are three observations from our experiments which help to reveal something of the nature of *Rph26* resistance. The first is that in the inheritance experiment, *Rph26* was found to display incomplete dominance, with the heterozygous genotypes (VB) possessing an intermediate level of resistance to *P. hordei* when compared with the parental (homozygous) genotypes (the presence or absence of the *H. bulboum* introgression on chromosome 1HL). This effect is generally caused by the different dosage of the resistance gene/allele; thus, there would be a higher amount of the resistance gene product (protein) in the homozygous resistant genotype possessing two copies of the gene/allele when compared with the heterozygous genotype carrying only one copy (Griffiths et al. 2000). A second observation is that the difference in response of 'Emir' and 200A12 to infection with *P. hordei* was very clear under low inoculum pressure

(Fig. 3); however, there was evidence from our experiments that the effect of *Rph26* was reduced under higher inoculum pressure. This suggests that *Rph26* resistance can become saturated at higher inoculum pressure. The third observation is that *Rph26* shows an increase in resistance over the course of plant development reaching full effect at flag leaf stage (data not shown). This is perhaps due to the expression of *Rph26* also increasing over plant development as with *Lr34* in wheat (Krattinger et al. 2009). These three observations collectively suggest that the level of *Rph26* expression is critical for effective resistance as gene/allele dosage (incomplete dominance effect) and plant developmental stage have large impacts on plant resistance. Although currently untested, it seems likely that the amount of inoculum required to saturate *Rph26* resistance would also change in response to allele/gene dosage and developmental stage.

For the genetic mapping of *Rph26*, F_3 lines with homozygous introgressions of reduced size were used to simplify the pathological characterization once F_2 interspecific recombinants were identified within the *H. bulbosum* introgression using flanking markers. These F_3 recombinant lines were characterized using a combination of visual assessments (uredinium count and ITs), statistical modelling (mean time and LP) and a fungal biomass assay. A qualitative assignment resistant or susceptible to all the recombinants was possible using only IT, which resulted in *Rph26* co-segregating with the markers CM_1143, CM_1144, CM_1191 and CM_1194.

The remaining pathological data (uredinium count at 6 DPI, uredinium count at 14 DPI, mean time, latency period and fungal biomass) gave continuous distributions, and thus, these five traits were mapped as separate QTLs. The peaks of all five QTLs were located at the proximal end of the original introgression between 0.10 and 0.11 cM and just proximal to the same cluster of four markers CM_1143, CM_1144, CM_1191 and CM_1194 at 0.15 cM. Twelve of the 19 recombinant lines in '200A12_F_3_Popn' possessed the 'BB' or 200A12 genotype for this cluster of markers, whilst the remaining seven recombinants possessed the 'VV' or 'Emir' genotype, and there were clear differences in the phenotypic distributions of these two genotypic classes. Nevertheless, there were some conflicting data for two lines, 377B_348_F_3 and 377B_708_F_3, which shared the same IT classification and identical marker genotype, but clearly differed for their uredinium counts and fungal biomass data (Table 1). These last two traits are intimately linked together, as the leaf material from the uredinium evaluation at 14 DPI was directly used in the fungal biomass assay. Further evaluation of these two lines against *P. hordei* in the second phenotypic experiment resulted in further ambiguity with 377B_708_F_3 appearing resistant in response to *P. hordei* in two of the replicates but susceptible in the third (Fig. 6). All lines were marker tested both before and after the experiment to eliminate the chance of mislabelling. There are at least two possible explanations for the unusual results observed for 377B_708_F_3. One hypothesis is that the *Rph26* resistance is conditioned by more than one closely linked gene and that the recombination event in 377B_708_F_3 has occurred within this locus making 377B_708_F_3 less resistant or more sensitive to the applied levels of inoculum or other changes to experimental conditions. An alternative hypothesis is that during the development of 377B_708_F_3, a mutation has occurred within *Rph26* which has reduced the efficacy of *Rph26* under certain experimental conditions. Further clarification with a larger number of 377B_708_F_3 may help to determine the correct phenotype of 377B_708_F_3 or to confirm whether this genotype has a particular sensitivity to changes in experimental conditions such as inoculum load. Clarity around the phenotype 377B_708_F_3 would also help to refine the location of *Rph26* to a much smaller genetic interval. For instance, if the line 377B_708_F_3 was confirmed to give a susceptible response to leaf rust, this would imply that the introgression 377B_348_F_3 extends further than 377B_708_F_3 in the proximal direction (to include *Rph26*). Additional marker development in this region could help to determine a much shorter interval for the gene *Rph26*.

Additional replicates of 377B_794_F_3 were included in the pathological characterization as a result of the F_2 genotyping (homozygous *H. bulbosum* genotype for CM_1186 and homozygous *H. vulgare* genotype for H31_14212), which revealed that 377B_794 was the product of two separate recombinant gametes (egg and pollen). The extra replicates were to protect against the chance that the smaller introgressions on each chromosome were of different sizes and that *Rph26* may be located in that intervening region and thus would be phenotypically segregating in the F_3 progeny. However, the pathological data (uredinium count at 6 DPI, IT, LP and mean time) of all of the 377B_794_F_3 plants were consistent with the results of the parental line 200A12. Three of the 24 plants did show slightly raised uredinium counts at 14 DPI, but all 24 plants gave the same IT as 200A12. The location of the *Rph26* resistance was determined to be proximal of this region, so no further analysis was undertaken for these plants.

This study shows the value of using multiple pathological assessments during the phenotyping of subtle resistance gene effects to ensure that phenotypic evaluations are consistent. Visual IT scores based on uredinium size, chlorosis and necrosis are external expressions of the host plant when its defence mechanisms are triggered by the rust pathogen penetrating into the apoplast and plant cells. Uredinium count can also be used to denote the pathological phenotypes of test lines; however, these data alone are unable to truly reflect the resistance responses of the plants, since other important characteristics like uredinium size are not taken

into account by uredinia counts. Perhaps for cereal rusts, where 97% of fungal biomass is located within the plant tissue (Ayliffe et al. 2013), a more quantitative assessment of internal fungal growth, such as fungal biomass, may reflect host response to *P. hordei* more accurately. Time permitting, the use of multiple pathological assessments will ultimately give a greater understanding of resistance gene effect.

The introgressed region containing the leaf rust resistance locus *Rph26* has been narrowed to within a 0.37-cM region or 50% of the genetic length of original introgression region. The goal of this study and other fine mapping studies, where agronomic alleles of interest have usually been confined to genomic regions of about 2–5 cM (Kumar et al. 2016; Qin et al. 2011; Schmalenbach et al. 2011; Zhang et al. 2013), is to have markers sufficiently close to the preferred gene/allele of the target trait to maximize the selection accuracy during marker-assisted breeding. The smallest proximal introgression containing *Rph26* has been identified in the lines 377B_794_F_3, 377B_953_F_3, 377B_1079_F_3, 377B_1122_F_3 and 377B_1296_F_3. The markers flanking this smaller introgressed region are tightly linked to the resistance locus and would facilitate the use of the *Rph26* in barley breeding through marker-assisted selection (MAS). Based on the 200A12 F_2 mapping population, the original introgression between the flanking markers CM_1186 and H31_14212 covered a genetic distance of 0.75 cM. In contrast, the Morex genome released by the International Barley Genome Sequencing Consortium (2012) indicated that the genetic distance between makers CM_1186 and H31_14212 was 6.5 cM. Thus, an eightfold reduction in recombination frequency was identified between *H. bulbosum* and barley compared with the barley intraspecific recombination frequency in the same region of chromosome 1HL. This high degree of suppressed recombination in *H. vulgare*/*H. bulbosum* introgression lines has also been reported by other studies, with up to a 14-fold reduction in interspecific recombinant frequency having been observed (Johnston et al. 2013; Ruge-Wehling et al. 2006). Suppressed recombination can be advantageous during MAS, as it reduces the likelihood of further recombination events between markers and traits but can also make it difficult to separate traits of interest from deleterious traits (linkage drag) in the same introgression. In order to map or clone genes of interest in any *H. bulbosum* introgression, a much larger mapping population is required compared with the same task within barley germplasm.

The closely linked markers identified in this study can be used to select the smallest introgression required to transfer *Rph26* into better adapted barley breeding lines. Removing extraneous portions of the introgression helps to reduce the probability of linkage drag caused by undomesticated genes or alleles that are genetically linked to the resistance locus. Although no unwanted linkage drag has been identified in 200A12, a significant yield penalty was previously identified and separated from *Rph22* and *Rym16*^{Hb} in an introgression of *H. bulbosum* on chromosome 2HL (Johnston et al. 2015). Although *Rph26* may not confer a high level of resistance to leaf rust on its own, the markers closely linked to *Rph26* will allow for its efficient combination with other resistance genes in the same genotype (pyramiding of multiple resistance alleles) within a short period of time (Park et al. 2015). Finding sources of potentially durable resistance to rust diseases in barley germplasm is an important objective for breeding programmes. However, the disease resistance conferred by a single, hypersensitive reaction-based, resistance gene (R-gene) has repeatedly proven non-durable as a result of the rapid evolution of new rust pathotypes which can overcome that gene (Park 2003). A better breeding strategy is the one that involves pyramiding multiple resistance genes into the same barley cultivar. The use of multiple resistance genes, especially involving different defence mechanisms, is likely to increase the effectiveness and durability of that resistance over time (Golegaonkar et al. 2009). With the help of closely linked markers, pyramiding of multiple resistance genes can be accelerated and validated through genotypic selection instead of the complicated and time-consuming selection based on subtle differences in the phenotypes for a combination of several resistance genes (Boopathi 2012). Based on the source of this resistance gene (*H. bulbosum*) and its chromosomal position on 1HL, we consider it to be a novel and detectable locus. Following consultation with Dr Frank Ordon, Julius Kuehn Institute, Germany and Dr Jerome Franckowiak, University of Minnesota, USA, it has been officially assigned the locus *Rph26* with the resistant allele *Rph26.ap*, in line with international protocols for disease resistance locus designation in barley.

Germplasm resources

A collection of 154 ILs (including 200A12) featuring chromosomal segments of *H. bulbosum* within a barley genetic background has been deposited with NordGen for preservation in the Svalbard Global Seed Vault (Pickering et al. 2010). All other genotypes discussed in this paper are available for distribution under material transfer agreement (MTA) from the corresponding author.

Author contribution statement Study was conceived by PAJ, XY, SCa, EEJ and SCh; plants materials were initially developed by RP; molecular markers were developed by HYK and PAJ; marker genotyping and fungal biomass were performed by XY and VM; linkage mapping by XY and PAJ; plant phenotyping was performed by XY; experimental designs, trial plans, statistical analyses and figures were done by RCB; and manuscript was written by PAJ and XY with contributions and editing from all authors.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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